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OBSERVATION

Identification of Plasmid-Mediated Tigecycline-Resistant Gene *tet*(X4) in *Enterobacter cloacae* from Pigs in China

Yiping Wu,[a,b] Ruowen He,[a,b] Mingyang Qin,[c] Yanxian Yang,[a,b] Jieyun Chen,[a,b] Yu Feng,[a,b] Xiaoxue Liang,[d] Wenbin Deng,[e] Xin Ding,[e] Li-Na Qin,[f] Kang Liao,[g] Yongqiang Yang,[a,b,e] Guo-Bao Tian[a,b,h]

[a]Department of Microbiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China
[b]Key Laboratory of Tropical Diseases Control, Sun Yat-sen University, Ministry of Education, Guangzhou, China
[c]School of Basic Medical Sciences, Xinxiang Medical University, Xinxiang, China
[d]School of Laboratory Medicine, Chengdu Medical College, Chengdu, China
[e]School of Pharmaceutical Sciences (Shenzhen), Sun Yat-sen University, Guangzhou, China
[f]Department of Histology and Embryology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China
[g]Department of Clinical Laboratory, the First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China
[h]School of Medicine, Xizang Minzu University, Xianyang, Shaanxi, China

Yiping Wu, Ruowen He, and Mingyang Qin contributed equally to this article. Author order was determined by drawing straws.

ABSTRACT Two *tet*(X4)-positive *Enterobacter cloacae* isolates TECL_1 and TECL_2 were isolated from pigs in China. S1-PFGE and Southern blotting showed that *tet*(X4) located on plasmids in the size of ~290 kb and ~190 kb in TECL_1 and TECL_2, respectively. Conjugation experiment demonstrated that the *tet*(X4)-harboring plasmid can transfer from the donor strain TECL_1 and TECL_2 to the recipient strain *Escherichia coli* J53, and the tigecycline resistance of transconjugants was increased by 128-fold and 64-fold compared with *E. coli* J53, respectively. We obtained the complete plasmid sequence of pTECL_2-190k-tetX4 (190,185 bp) from *E. cloacae* TECL_2 and found that the plasmid was a hybrid plasmid with replicon types of IncFIA, IncHI1A and IncHI1B. We further analyzed 85 *tet*(X4)-carrying plasmids in the public database and clarified that pTECL_2-190k-tetX4-like plasmid was widespread in multiple species of Enterobacteriaceae.

IMPORTANCE We identified two *tet*(X4)-positive *E. cloacae* isolates, which has not been previously reported. We obtained the complete sequence of pTECL_2-190k-tetX4 and found that it was a hybrid plasmid with multiple replicon types, including IncFIA, IncHI1A and IncHI1B. By comparing all the known *tet*(X4)-carrying plasmids, we found that pTECL_2-190k-tetX4-like plasmid has been disseminated across various species in China. Our study expanded the identification of *tet*(X4)-positive species and emphasized that pTECL_2-190k-tetX4-like plasmid has spread widely in various species.

KEYWORDS *tet*(X4), tigecycline resistance, *Enterobacter cloacae*

Enterobacter cloacae is one of the members of Enterobacteriaceae, which was reported as an important opportunistic microbial pathogen for a broad range of hospital-acquired infections (1). Tigecycline is a last-resort antibiotic for the treatment of life-threatening infections caused by multidrug-resistant bacteria, such as carbapenem-resistant Enterobacteriaceae (2). In recent years, the tigecycline resistance gene *tet*(X) has been reported to mediate high-level resistance to all tetracycline antibiotics in isolates from animals, humans and the environment, posing a significant risk to public health (3–5). In 2019, a plasmid-borne high-level tigecycline resistance gene *tet*(X) was identified (6). Herein, we identified two *E. cloacae* isolates harboring plasmid-mediated *tet*(X) gene, which has not been reported, and further analyzed the genetic context of the *tet*(X4)-carrying plasmid pTECL_2-190k-tetX4.

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Address correspondence to Guo-Bao Tian, tiangb@mail.sysu.edu.cn, Yongqiang Yang, yangyongqiang2000@126.com, or Kang Liao, liaokang@mail.sysu.edu.cn.

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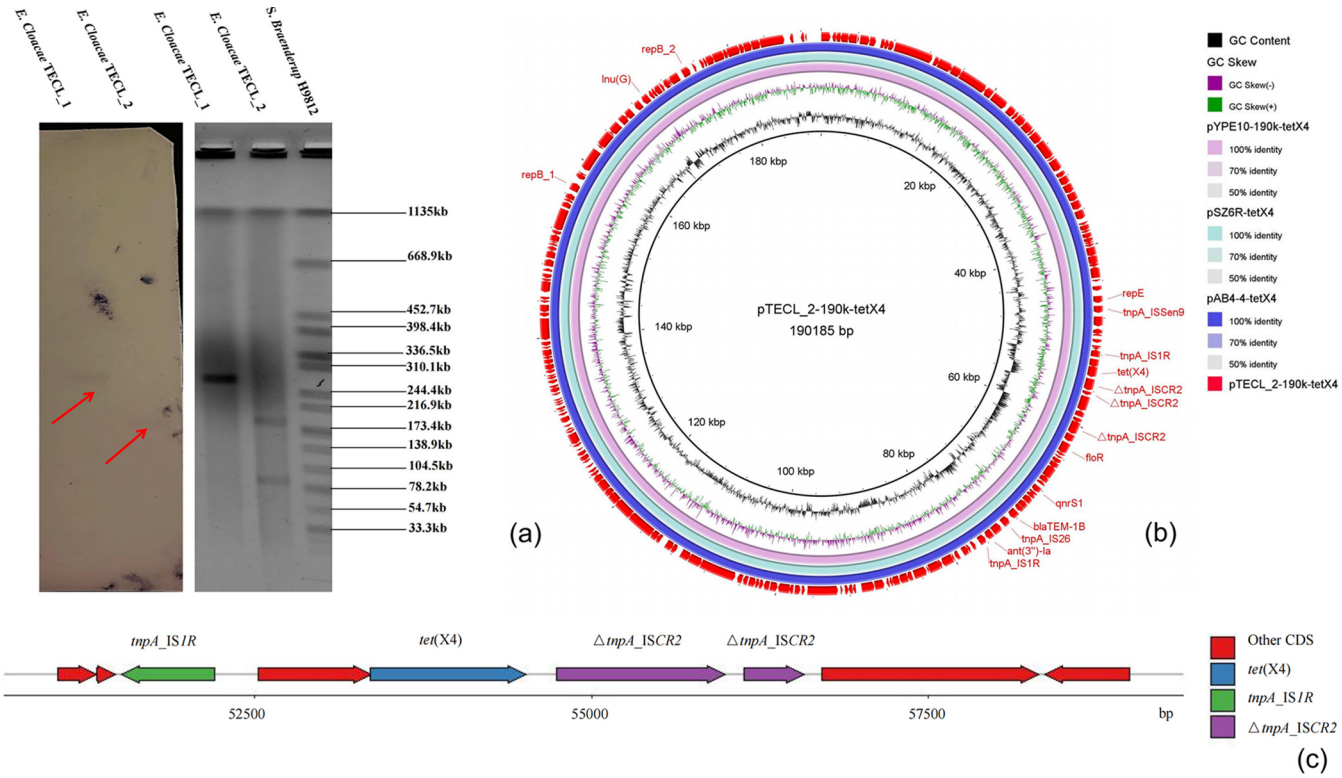
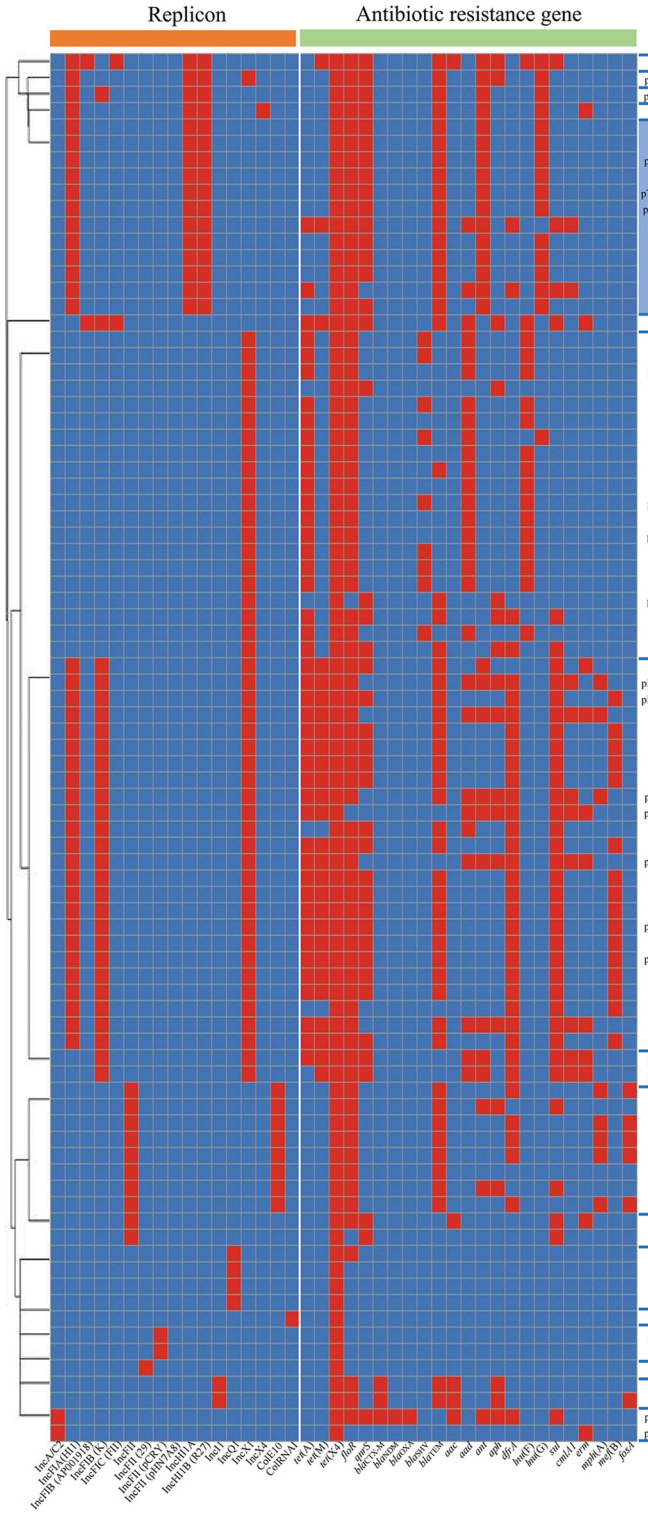


FIG 1 The plasmid structure of pTECL_2-190k-tetX4. (a) The location of *tet*(X4) in *E. cloacae* isolates TECL_1 and TECL_2 by S1-PFGE and Southern blotting. *Salmonella Braenderup* strain H9812 was used as the marker by *Xba*I enzyme digestion. (b) The circular genetic map of pTECL_2-190k-tetX4, pYPE10-190k-tetX4 (GenBank accession No. CP041449.1), pSZ6R-tetX4 (GenBank accession No. MW940627.1) and pAB4-4-tetX4 (GenBank accession No. MW940615.1). The arrows on the outer circle represent the genes of replicon, antibiotic resistance, and transposase. The three middle circles show the similarity of three plasmids harboring *tet*(X4) with pTECL_2-190k-tetX4. The inner arc represents GC skew curve and the next represents GC contents. (c) Major structural features of the *tet*(X4) gene. The blue arrow represents *tet*(X4). The green arrow represents the transposase of the IS*1R* element and the transposase of the ΔIS*CR2* element is purple. The number marked on the ruler at the bottom of the picture corresponds to the nucleotide position on the plasmid.

We collected 590 nonduplicate samples, including 475 pig nasal swabs, 67 pig anal swabs and 48 staff skin swabs from a pig farm and a slaughterhouse in Guangdong Province. Then colonies were selected from BHI plate containing 4 mg/L tigecycline after preinoculation and screened for *tet*(X4) variants by PCR (7). Finally, we identified two *E. cloacae* strains TECL_1 and TECL_2 carrying *tet*(X4). MICs of 14 antimicrobial agents for strains were determined (Table S1). Both strains were resistant to tigecycline, tetracycline, rifamycin, ampicillin, chloramphenicol and ciprofloxacin. In addition, TECL_1 was resistant to fosfomycin; TECL_2 was resistant to ceftazidime, colistin sulfate, cefotaxime, gentamicin, and trimethoprim-sulfamethoxazole. Then the two *E. cloacae* isolates were subjected to genomic DNA extraction and whole-genome sequencing. The sequencing reads were assembled into contigs using SPAdes version 3.10 (8). Antibiotic resistance genes (ARGs) were predicted using ResFinder v3.2 (9). It showed that TECL_1 harbored nine ARGs including *tet*(X4), *tet*(M), *aadA2*, *aadA22*, bla_{TEM-1B}, *qnrS1*, *lnu*(G), *lnu*(F) and *floR*. TECL_2 carried 21 ARGs including *tet*(X4), *tet*(A), *ant(3")-Ia*, *aadA16*, *aac(6')-Ib-cr*, *aac(3)-IId*, *aph(6)-Id*, *aph(3')-Ia*, *aph(3")-Ib*, bla_{CTX-M}, bla_{CMH-3}, *oqxA*, *oqxB*, *qnrS1*, *lnu*(G), *fosA*, *mph*(A), *sul1*, *sul2*, *arr-3* and *dfrA27*.

Previous studies reported that *tet*(X4) is mostly located on the plasmid in Enterobacteriaceae (10, 11). To determine the transferability of *tet*(X4)-harboring plasmids in *E. cloacae* isolates, we performed the conjugation experiment. It showed that *tet*(X4) could be successfully transferred from TECL_1 and TECL_2 into the recipient *E. coli* J53 by filter mating. The transconjugants J53/pTECL_1-290k-tetX4 and J53/pTECL_2-190k-tetX4 were resistant to tigecycline with MIC values of 32 and 16 mg/L, respectively. The tigecycline resistance of two transconjugants was increased by 128-fold and 64-fold compared with *E. coli* J53 (Table S1). Then PCR and Sanger sequencing demonstrated that the



Metadata

Plasmid	GenBank accession number	Size(bp)	Organism	Region	Source	Collection date	Group
pSY3626C1_315k	CP059044.1	315984	*Escherichia coli*	China:Sichuan	chicken	2020	group1
pRW7-1_235k_tetX	MT219825.1	235947	*Escherichia coli*	China	wastewater	2019	group2
pRT18-1_294k_tetX	MT219824.1	294397	*Escherichia coli*	China	carcass	2019	group3
pYSP8-1	CP037911.1	239511	*Escherichia coli*	China:Guangdong	swine feces	2017	group4
pAB4-4-tetX4	MW940615.1	193385	*Klebsiella pneumoniae*	China	pork	2019	group5
p1919D62-1	CP046007.1	186058	*Escherichia coli*	China:Henan	swine feces	2019	
pYPE10-190k-tetX4	CP041449.1	190128	*Escherichia coli*	China: Jiangsu	pork	2019	
pSZ6R-tetX4	MW940627.1	191434	*Citrobacter braakii*	China	pork	2019	
pTECL_2-190k-tetX4	MZ773210.1	190185	*Enterobacter cloacae*	China:Guangdong	pig nasal swab	2020	
pSY3626_190k_tetX	CP059284.1	190381	*Escherichia coli*	China: Sichuan	chicken	2020	
pSal21GXH-tetX4	CP060586.1	256017	*Salmonella enterica*	China: Guangxi	human stool	2016	
pT28R-1	CP049354.1	193098	*Escherichia coli*	China: Henan	pet dog feces	2019	
p2019XSD11-190	MN101856.1	190390	*Escherichia coli*	China	pork	2019	
pT16R-1	CP046717.1	190391	*Escherichia coli*	China: Henan	pet dog feces	2019	
p1919D3-1	CP046004.1	219101	*Escherichia coli*	China:Henan	swine feces	2019	
pSX8G-tetX4	MW940625.1	190382	*Escherichia sp.*	China	pork	2019	
p47EC	MK134376.1	170312	*Escherichia coli*	China	swine feces	2018	group6
pSX5G-tetX4	MW940624.1	57105	*Escherichia sp*	China	pork	2019	group7
pYY76-1-2	CP040929.1	57104	*Escherichia coli*	China:Guangdong	cow feces	2018	
pRB3-1_31K_tetX	MT197111.1	31287	*Escherichia coli*	China	ground blood	2019	
pSC4R-tetX4	MW940620.1	55809	*Citrobacter sp.*	China	pork	2019	
pHS19-2-tetX4	MW940619.1	57105	*Klebsiella sp.*	China	pork	2019	
pEC05-X4	MN436006.1	32181	*Escherichia coli*	China	pig slaughterhouse	2019	
p1916D18-1	CP045998.1	59353	*Escherichia coli*	China: Henan	swine feces	2019	
pSZ5R-tetX4	MW940626.1	31287	*Escherichia sp*	China	pork	2019	
pEC931_tetX	CP049121.1	50626	*Escherichia coli*	China: Zhejiang	human urine sample	2017	
pEC12-X4	MN436007.1	32074	*Escherichia coli*	China	pig slaughterhouse	2019	
pHNCF11W-tetX4	CP053047.1	57105	*Escherichia fergusonii*	China: Henan	chicken feces	2019	
pSH9W-tetX4	MW940622.1	31587	*Escherichia sp.*	China	pork	2019	
pRF45-1_31k_tetX	MT219821.1	31287	*Escherichia coli*	China	swine feces	2019	
pSZ11R-tetX4	MW940629.1	56939	*Escherichia sp*	China	pork	2019	
pHS10-1-tetX4	MW940618.1	48817	*Escherichia sp*	China	pork	2019	
pHN10R-tetX4	MW940616.1	57096	*Escherichia sp*	China	pork	2019	
pRF14-1_50k_tetX	MT219822.1	50518	*Escherichia coli*	China	swine feces	2019	
pCD74-2-2	CP050046.1	44690	*Escherichia coli*	China:Sichuan	chicken	2019	
p1916D6-2	CP046002.1	59351	*Escherichia coli*	China: Henan	swine feces	2019	
pCD58-3-1	CP050037.1	44691	*Escherichia coli*	China:Sichuan	chicken	2019	
pHN13R-tetX4	MZ054178.1	169783	*Escherichia coli*	China	pork	2019	group8
pRF148-1_119k_tetX	MT219818.1	119185	*Escherichia coli*	China	swine feces	2019	
pRF148-2_101k_tetX	MT219817.1	101373	*Escherichia coli*	China	swine feces	2019	
pSZ10R-tetX4	MW940628.1	130185	*Escherichia sp.*	China	pork	2019	
pNT1N28-tetX4	CP075473.1	148421	*Escherichia coli*	China	pig farm	2018	
pNT1F25-tetX4	CP075471.1	122563	*Escherichia coli*	China	pig farm	2018	
pSTB20-1T	CP050174.1	97366	*Escherichia coli*	China: Jiangsu	swine feces	2017	
pNT1F34-tetX4	CP075486.1	130047	*Escherichia coli*	China	pig farm	2018	
pRW8-1_122k_tetX	MT219826.1	122608	*Escherichia coli*	China	wastewater	2019	
pRF10-1_119k_tetX	MT219823.1	119011	*Escherichia coli*	China	swine feces	2019	
pCD63-2-1	CP050041.1	89748	*Escherichia coli*	China:Sichuan	broiler chicken	2019	
pNT1N25-tetX4	CP075485.1	115741	*Escherichia coli*	China	pig farm	2018	
pRF52-1_119k_tetX	MT219819.1	119180	*Escherichia coli*	China	swine feces	2019	
pNT1W22-tetX4	CP075470.1	101476	*Escherichia coli*	China	pig farm	2018	
pNT1F10-tetX4	CP075463.1	97500	*Escherichia coli*	China	pig farm	2018	
pNT1N34-tetX4	CP075467.1	103081	*Escherichia coli*	China	pig farm	2018	
pRF108-2_97k_tetX	MT219820.1	97526	*Escherichia coli*	China	swine feces	2019	
pNT1F31-tetX4	CP045188.1	115253	*Escherichia coli*	China: Jiangsu	swine feces	2018	
pYPE12-101k-tetX4	CP041443.1	101987	*Escherichia coli*	China: Jiangsu	pork	2019	
pNT1W25-tetX4	CP075479.1	102128	*Escherichia coli*	China	pig farm	2018	
p54-tetX	CP041286.1	102347	*Escherichia coli*	China	cow feces	2018	
pYPE3-92k-tetX4	CP041453.1	92973	*Escherichia coli*	China: Jiangsu	pork	2019	
pG3X16-2-3	CP038140.1	138950	*Escherichia coli*	China: Guangxi	swine feces	2017	
pNT1N31-tetX4	CP075481.1	103246	*Escherichia coli*	China	pig farm	2018	
pZF31-tetX-119kb	CP047460.1	118248	*Escherichia coli*	China: Jiangsu	swine feces	2018	group9
pZF34-tetX-114kb	CP047466.1	114813	*Escherichia coli*	China: Jiangsu	swine feces	2018	
pPK5086-tetX	CP080371.1	100261	*Escherichia coli*	Pakistan: Faisalabad	human	2019	group10
pPK5074-tetX	CP072807.1	110313	*Escherichia coli*	Pakistan: Faisalabad	human urine sample	2019	
pPK8261-tetX	CP080156.1	119081	*Escherichia coli*	Pakistan: Faisalabad	chicken	2020	
pPK8277-tetX	CP080134.1	101760	*Escherichia coli*	Pakistan: Faisalabad	chicken	2020	
pPK8275-tetX	CP080164.1	119081	*Escherichia coli*	Pakistan: Faisalabad	chicken	2020	
pPK8568-tetX	CP080129.1	91844	*Escherichia coli*	Pakistan: Faisalabad	chicken	2021	
pPK8217-tetX	CP080124.1	105596	*Escherichia coli*	Pakistan: Faisalabad	chicken	2021	
pPK8566-tetX	CP080175.1	111556	*Escherichia coli*	Pakistan: Faisalabad	chicken	2020	
pAB12-1-tetX4	MZ054177.1	174429	*Escherichia coli*	China	pork	2019	group11
p2GS3-tetX4	MW940614.1	91582	*Escherichia sp.*	China	pork	2019	
pPK8276-tetX	CP080147.1	12396	*Escherichia coli*	Pakistan: Faisalabad	chicken	2020	group12
pLHM10-1	CP037909.1	12783	*Escherichia coli*	China:Jiangxi	swine feces	2017	
pSH12R-tetX4	MW940623.1	12806	*Escherichia sp.*	China	pork	2019	
pHS2-1-tetX4	MW940617.1	12805	*Escherichia sp.*	China	pork	2019	
p16EC-9K	MN381965.1	9928	*Escherichia coli*	China	swine feces	2018	group13
pNTT31XS-tetX4	CP077430.1	78159	*Klebsiella aerogenes*	China: Jiangsu	porcine intestinal contents	2020	group14
pSDP9R-tetX4	MW940621.1	78159	*Klebsiella sp.*	China	pork	2019	
pPK8241-tetX	CP080140.1	66580	*Escherichia coli*	Pakistan: Faisalabad	chicken	2020	group15
p2EC1-1	CP047572.1	127645	*Escherichia coli*	Singapore	human feces	2019	group16
p94EC-2	CP047578.1	134426	*Escherichia coli*	Singapore	human feces	2019	
pNUITM-VK5_mdr	LC633285.1	240546	*Klebsiella aerogenes*	Viet Nam: Hanoi	wastewater	2021	group17
pRF173-1_87k_tetX	MT219816.1	87445	*Escherichia coli*	China	swine feces	2019	

FIG 2 The comparison of the publicly available plasmids carrying *tet*(X4). The average-linkage clustering method was used to cluster 85 plasmids carrying *tet*(X4) according to the replicon type. The groups were separated by blue horizontal lines in the figure. The prominent part of the blue block is the group which pTECL_2-190k-tetX4 belongs. The distribution of all replicon types and antibiotic resistance genes were presented by heatmap. In terms of whether the corresponding plasmid replicon and antibiotic resistance genes is present in the plasmid, red represents presence and blue represents absence. The metadata of plasmids is shown on the right of figure, including the name, GenBank accession number, size, and source of plasmids.

transconjugants carried *tet*(X4). Furthermore, S1-PFGE and Southern blotting hybridization revealed that *tet*(X4) located on plasmids in the size of ~290 kb and ~190 kb in TECL_1 and TECL_2, respectively (Fig. 1a). The results proved that *tet*(X4) was located on the plasmid of *E. cloacae* isolates, and could be transmitted to other species, causing the spread of tigecycline resistance in Enterobacteriaceae.

To determine the complete sequences of pTECL_2-190k-tetX4, we combined the sequencing data from the genomic DNA and the plasmids and closed predicted gaps within the sequences by PCR and Sanger sequencing using primers listed in Table S2. Finally, we obtained the complete sequence of pTECL_2-190k-tetX4 from *E. cloacae* strain TECL_2. The sequence was analyzed using the method mentioned in the previous article (12). pTECL_2-190k-tetX4 was a 190,185 bp plasmid with three replicon types IncFIA, IncHI1A and IncHI1B, and encoded 220 predicted ORFs (Fig. 1b). pTECL_2-190k-tetX4 showed a mosaic structure harboring six ARGs, including *tet*(X4) along with *ant*(3')-*Ia*, bla_{TEM-1B}, *lnu*(G), *floR* and *qnrS1*. The *tet*(X4) gene, was flanked by a complete IS*1R* element and a truncated IS*CR2* element. This IS*1R* element was located at 1099 bp upstream of *tet*(X4). The downstream region of *tet*(X4) was a 223 bp fragment encoding the transposase of IS*CR2* element. There is a 136 bp fragment insertion resulting in the truncation of IS*CR2* element. (Fig. 1c). BLASTn of pTECL_2-190k-tetX4 against the nr database retrieved similar plasmids from different hosts. The plasmid pTECL_2-190-tetX4 was highly similar with pYPE10-190k-tetX4 isolated from *E. coli* strain YPE10 (CP041449.1, 99.96% identity at 100% coverage) (13), pSZ6R-tetX4 isolated from *Citrobacter braakii* strain SZ6R (MW940627.1, 99.92% identity at 100% coverage) (14) and pAB4-4-tetX4 isolated from *Klebsiella pneumoniae* strain AB4-4 (MW940615.1, 99.92% identity at 100% coverage) (14). The result indicated that the pTECL_2-190k-tetX4-like plasmid might have been widely spread in different species of Enterobacteriaceae.

It was reported that the plasmid carrying *tet*(X4) showed structure diversity (15). We compared all the known *tet*(X4)-carrying plasmids and tried to clarify the prevalence of plasmids carrying *tet*(X4), especially the pTECL_2-190k-tetX4-like plasmid. BLASTn of the *tet*(X4) gene identified 90 publicly available *tet*(X4)-carrying plasmids, as of 7 August 2021. We excluded three transconjugative plasmids, one plasmid with the sequence of repeated uploads and two plasmids without replicon sequence. The remaining 84 complete plasmid sequences were compared with pTECL_2-190k-tetX4 (Fig. 2). The 85 plasmids carrying *tet*(X4) were clustered 17 types of plasmid group by the different replicon types. pTECL_2-190k-tetX4-like plasmids (at least 99.91% identity at 92% coverage with pTECL_2-190k-tetX4) are one of the dominant type of plasmids harboring *tet*(X4) (n = 12). The size of the plasmids is about 200 kb. Besides *tet*(X4), multiple ARGs were co-existed in these plasmids such as *floR*, *qnrS1*, bla_{TEM-1B}, *ant(3")-Ia* and *lnu*(F) (Table S3). Strains carrying pTECL_2-190k-tetX4-like plasmid have been found from swine, pet dog and chicken in China, indicating that this type of plasmid has disseminated widely in animals in China (Fig. 2). *E. cloacae* TECL_2 was isolated from a pig farm in Guangdong Province, indicating the transmission range of the plasmid in China has been further expanded. In addition, it is noteworthy that the host bacteria of this plasmid are diverse, such as *E. coli*, *E. cloacae*, *S. enterica*, *K. pneumoniae* and *C. braa*kii, indicating this plasmid has strong host adaptability.

In conclusion, the identification of two *tet*(X4)-positive *E. cloacae* isolates indicates that the host range of *tet*(X4) has been further expanded. In addition, the widespread of pTECL_2-190k-tetX4-like plasmid in Enterobacteriaceae must be concerned.

Data availability. This complete sequence for pTECL_2-190k-tetX4 has been deposited in GenBank under the accession number MZ773210.1.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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The authors report no conflicts of interest in this work.

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